MOLECULAR RECOGNITION IN GELS, MONOLAYERS, AND SOLIDS

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This paper summarizes research in two areas of molecular recognition: affinity polymers and molecular self-assembly. We illustrate these areas by examples drawn from affinity gel electrophoresis, soluble synthetic macromolecular inhibitors of binding of influenza virus to erythrocytes (*1*), protein adsorption on self-assembled monolayers (*2,3*), and self-assembling hydrogen-bonded molecular aggregates (*4–6*).

Molecular Recognition in Gels, Monolayers, and Solids

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This paper describes work in four areas: affinity electrophoresis of carbonic anhydrase in cross-linked polyacrylamide-derived gels containing immobilized derivatives of aryl sulfonamides; inhibition of the hemagglutination of erythrocytes induced by influenza virus using water-soluble polyacrylamides bearing sialic acid groups; the application of self-assembled monolayers (SAMs) of alkyl thiolates on gold to the study of protein adsorption on organic surfaces; and the use of networks of hydrogen bonds to generate new classes of non-covalently assembled organic materials, both in solution and in crystals.

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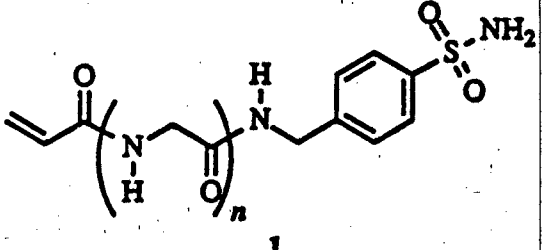
This paper summarizes research in two areas of molecular recognition: affinity polymers and molecular self-assembly. We illustrate these areas by examples drawn from affinity gel electrophoresis, soluble synthetic macromolecular inhibitors of binding of influenza virus to erythrocytes (*1*), protein adsorption on self-assembled monolayers (*2,3*), and self-assembling hydrogen-bonded molecular aggregates (*4–6*).

Affinity Polymers: Molecular Recognition in Gels

Affinity gel electrophoresis (AGE) uses the biospecific equilibrium binding of a protein to an immobilized ligand to reduce the electrophoretic mobility of that protein selectively. AGE is a useful technique for studying receptor–ligand interactions (*7*). It combines the selectivity of affinity chromatography with the high sensitivity of gel electrophoresis. AGE allows both the qualitative examination of the specificity of binding between protein and covalently immobilized ligand and the quantitative determination of the dissociation constant of the protein–ligand complex. By observing how the dissociation constant changes with the structure of the ligand, it is possible to probe the chemical characteristics and topology of the ligand-binding site.

We chose carbonic anhydrase B (CAB, E.C.4.2.1.1) as a model protein for our initial studies of AGE. CAB is a well-characterized protein (*8–11*). It is inhibited by a number of aryl sulfonamides, with dissociation constants ranging from 10^{-6} to 10^{-8} M(*11*). The active site of the enzyme is known from X-ray crystallography and can be described qualitatively as being located at the bottom of a conical pocket approximately 15 Å deep and 15 Å wide.

In order to test the sensitivity of AGE to the topology of a binding pocket, we prepared the series of glycyl-linked monomers **1** and formed gels by copolymerizing them in different concen-



1

1a $n = 0$
1b $n = 1$
1c $n = 2$
1d $n = 3$
1e $n = 4$
1f $n = 5$
1g $n = 6$
1h $n = 7$

trations with acrylamide and crosslinking agent. These gels were used as the stationary phase in electrophoresis experiments. The retention ratio, R_f, of CAB on electrophoresis in these gels was a function of the concentration of immobilized sulfonamide in the gel, [L], as illustrated in Figure 1 for gels based on poly(**1f**-*co*-acrylamide). The retention ratio is related to the dissociation constant, K_d, by equation 1 (*7*). When $[L]R_f$ is plotted as a function of R_f, both the slope and the $[L]R_f$-intercept

$$[L]R_f = K_d - K_d R_f \quad (1)$$

give the value of K_d. Figure 2 shows the measured values of K_d for the affinity ligands **1**, as a function of the number of glycine residues in the spacer. From these data, we conclude that the binding pocket of CAB is insensitive to linking chains longer than three glycine residues. Use of a linker connecting the sulfonamide to the polymer backbone shorter than $(gly)_4$ gives an apparent K_d that is larger than the solution value, reflecting (we presume) unfavorable steric interactions between the protein and the backbone. This value is in agreement with our estimate from the crystallographic dimensions of the binding pocket.

Affinity-Polymer Inhibition of Influenza-Induced Agglutination of Erythrocytes. We have also begun to design soluble, polymeric affinity ligands to interact with proteins on biological surfaces. We have explored the inhibition of the agglutination of erythrocytes induced by influenza virus in

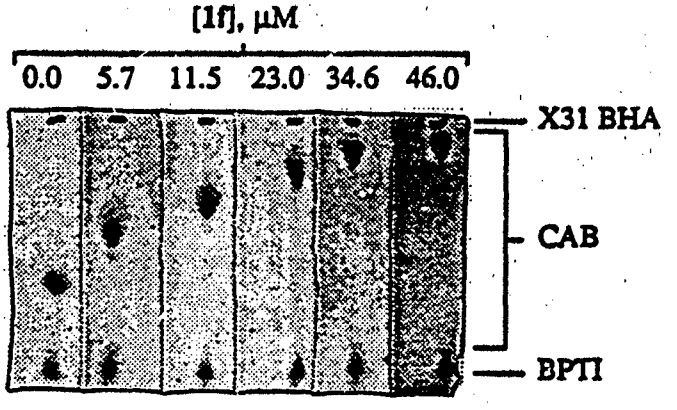


Figure 1. Affinity electrophoresis of bovine carbonic anhydrase B (CAB) on polyacrylamide slab gels containing various concentrations of affinity ligand 1f. Bovine pancreatic trypsin inhibitor (BPTI) and the bromelain-released hemagglutinin of influenza virus X-31 (X31 BHA) were used as internal standards.

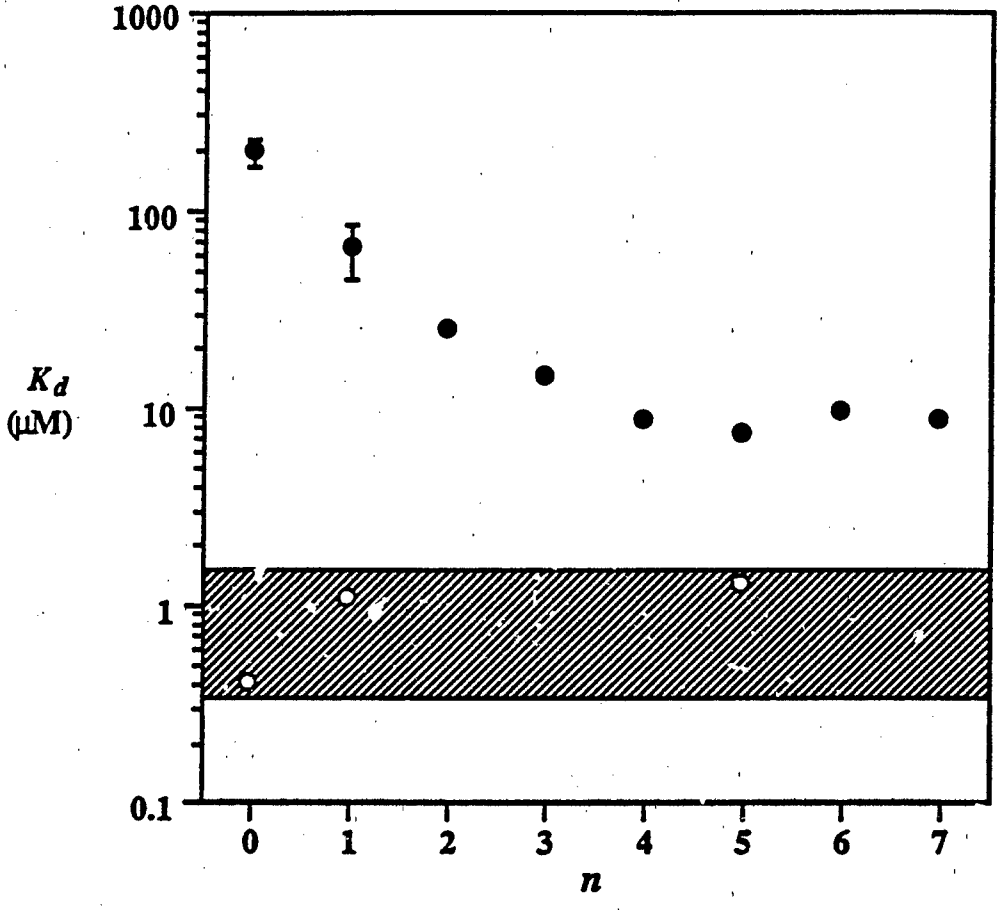
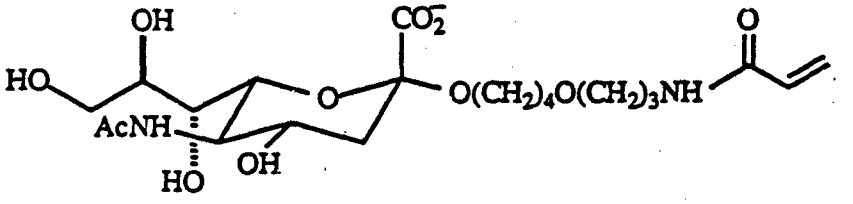


Figure 2. Dependence of the dissociation constants, K_d, of complexes of bovine carbonic anhydrase B (CAB) and immobilized (filled circles) or soluble (hollow circles) affinity ligands 1 on the number of glycine residues, n, in the ligand.

greatest detail. Hemagglutinin (HA) present on the viral surface binds to sialic acid (SA) residues on glycoproteins and glycolipids located at the surface of the cell (*12–14*). Unlike the tight-binding ($K_d = 10^{-6}$–10^{-8} M) CAB–sulfonamide system, the HA–SA complex is weakly bound ($K_d = 2$ mM) (*15*). Although there is no corresponding value for the binding of virus to erythrocyte, the binding of genetically altered firbroblasts expressing HA on their surface to erythrocytes has a substantially lower dissociation constant ($K_d \cong 7 \times 10^{-10}$ M) (*16*). We and others believe that the difference in strength between the interactions of HA with sialic acid and of influenza virus with erythrocyte can be traced to the polyvalency of the latter (*17–20*). We wished to test the hypothesis that an appropriate polyvalent molecule presenting many sialic acid residues to the virus would be an effective inhibitor of the binding of influenza to erythrocytes.

The naturally occurring hemagglutination inhibitors are structurally complex glycoproteins (*21, 22*), and rather than attempting to mimic these proteins (*23*), we chose to include sialic acid residues in acrylamide-derived polymers. We hoped that the flexibility of the acrylamide backbone would allow multiple sialic acid residues per polymer chain to bind to the surface of the virus particle, and that this multipoint attachment would result in strong inhibition of the binding of virus to erythrocytes. Acrylamide-derived polymers are well suited for this purpose, since they can be prepared easily, their structures can be varied readily, and they are water-soluble.

We synthesized monomer **2** and copolymerized it with a number of acrylamide monomers (3a-g). Figure 3 shows the inhibition constant of the soluble polymer, K_i, determined by a hemag-



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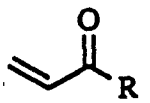


3

3a R = NH_2
3b R = $NHCH_2OH$
3c R = $N(CH_3)_2$
3d R = $NHC(CH_2OH)_3$
3e R = $NH(CH_2)_3O(CH_2)_4O$-β-Glc
3f R = $NH(CH_2)_3O(CH_2)_3CO_2^-$
3g R = $NH(CH_2)_6NH_3^+$

glutination assay, as a function of the mole fraction, χ_{SA}, of **2** in the mixture of **2** and **3a** used to form the polymer (*1*). The values of K_i were calculated on the basis of sialic acid groups in solution. Polymers having values of $K_i > 0.625$ mM (the horizontal line in Figure 3) were not examined quantitatively; the hollow points represent upper limits. The values of K_i for proteins and analogs of sialic acid were obtained from the literature and are shown on the right. The strongest inhibition occurred over a broad range of χ_{SA} (0.2–0.6) and was within an order of magnitude of the best naturally occurring inhibitors. Copolymers derived from the other co-monomers (3b-g) showed little difference in inhibition but were less water-soluble than those derived from 3a. Copolymers derived from analogs of **2** containing shorter spacers, however, showed significantly lower inhibition constants. We do not yet understand why sialic acid residues bound to short spacers are less efficient

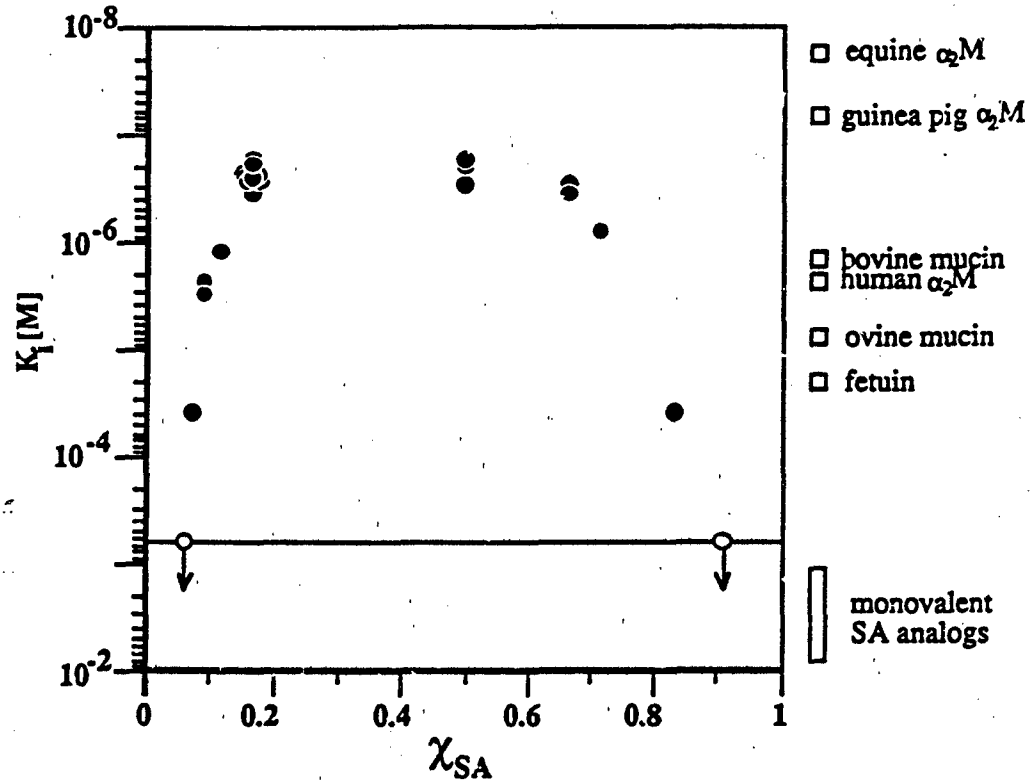


Figure 3. Inhibition of hemagglutination of erythrocytes by poly(2-*co*-acrylamide). (Reproduced with permission from ref. 1. Copyright 1991, American Chemical Society.)

inhibitors than those linked to long spacers, but the X-ray structure of hemagglutinin shows that the binding site is *not* in a pocket. This observation suggests that the change in inhibition with the length of the spacer may arise from changes in the structure of the affinity polymer itself, or from interaction between the HA and the polymer backbone. Similar results were found by other groups (*24,25*). We are currently working to optimize the performance of these polymers and to determine the relationship between their structures and their inhibition constants.

Self-Assembly: Molecular Recognition in Monolayers and Solids

The term "self-assembly" is used to describe a variety of processes, all of which involve the spontaneous organization of dispersed molecules into an ensemble with a defined structure. Self-assembled structures are ubiquitous in nature: the double helix of DNA, many multi-unit enzymes, structural proteins, ribosomes, and viruses assemble spontaneously into their native structures from

solutions of their constituent parts (*26*).

Self-assembly is also a practical synthetic strategy in the laboratory (albeit at a simpler level than in nature!). For example, long-chain surfactants with terminal groups capable of bonding to solid surfaces (head groups) self-assemble into ordered, oriented monomolecular films when solutions of the surfactants contact the solid surfaces. Such self-assembled monolayers (SAMs) are known for alkanoic acids on a variety of metal oxides (*27*); trichlorosilanes on oxide surfaces (*28*), such as silica (*29–32*) and alumina (*33*); alkanethiols, dialkyl sulfides, and dialkyl disulfides on gold, silver, and copper (*34*); and alkyl isonitriles on platinum (*35*). The monolayer–air interface of a SAM comprises principally an ordered array of the tail group (the end of the molecule opposite from the Au–S interface). By synthetic variation in the tail groups, SAMs can be prepared that exhibit a wide variety of properties (*36*).

In this section, we describe first the use of SAMs as model systems for studying the adsorption of proteins on organic surfaces. We then turn to an example of a different strategy for self-assembly: the use of hydrogen-bonded networks to prepare large, self-assembling complexes.

Self-Assembled Monolayers as Substrates for Studying the Mechanisms of Adsorption of Proteins to Man-Made Surfaces. SAMs formed by the adsorption of alkanethiols onto gold have received considerable attention in our laboratories (*34*). Two attractive features of this form of SAM are the variety of polar functional groups that are compatible with Au–S binding and the ease of preparing SAMs containing mixtures of tail groups from solutions containing mixtures of different alkanethiols (*36*).

We have used mixed SAMs to model polymer surfaces that contain poly(ethylene glycol) and different amounts of hydrophobic material (*3*). Figure 4 suggests schematically the structure of the monolayer–water interface of one of the SAMs. The tail group is flexible and drawn roughly to scale. We immerse the SAMs in solutions of proteins and observe (by ellipsometry and X-ray photoelectron spectroscopy) the amount of protein that is retained on the SAM after rinsing it with water (*2*).

Figure 5 shows the amount of fibrinogen adsorbed to SAMs containing a mixture of two

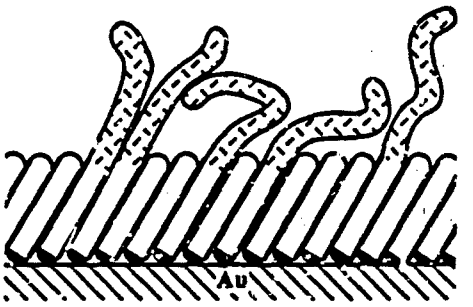


Figure 4. A schematic representation of mixed SAMs of 4 and 5e. (Reproduced with permission from ref. 2. Copyright 1991 American Association for the Advancement of Science.)

coadsorbed thiolates ("mixed SAMs") prepared from various mixtures of 4 and 5. The thicknesses of the adsorbed films of protein, d, were determined by ellipsometry. The values of χ represent the

$CH_3(CH_2)_{10}SH$ — 4

$RO(CH_2CH_2O)_n(CH_2)_{11}SH$ — 5

5a $n = 0$
5b $n = 1$
5c $n = 2$
5d $n = 4$
5e $n = 6$
} R = H

5f $n_{ave} = 17$ — $R = CH_3$

surface mole fraction of 5 in the SAM, as determined by XPS. Each datum represents the average of three measurements taken on one SAM. The scatter in each average falls within the size of the symbol used to represent it. The results are reproducible: the curves for $n = 0$, 2, 4, and 6 represent two independent sets of experiments each. The data have been offset in increments of 20 Å for clarity. The dashed lines on the right side of the graph represent the location of $d = 0$ Å for each set of experiments; the symbols to the right of the dashed lines indicate to which set of data each zero line applies.

Two observations can be made from these data: First, there is a qualitative difference between the adsorption of fibrinogen to SAMs containing hydroxyl- or mono(ethylene glycol)-terminated chains and to SAMs containing di(ethylene glycol)- or oligo(ethylene glycol)-terminated chains. The advancing contact angles of water upon SAMs formed from 5a, 5b, 5c, and oligo(ethylene glycol)-terminated alkanethiols are 0°(*34*), 20°, 27°, and 33° (*3*), respectively (unpublished results unless otherwise noted). We do not yet know whether or how these two sets of observations are related.

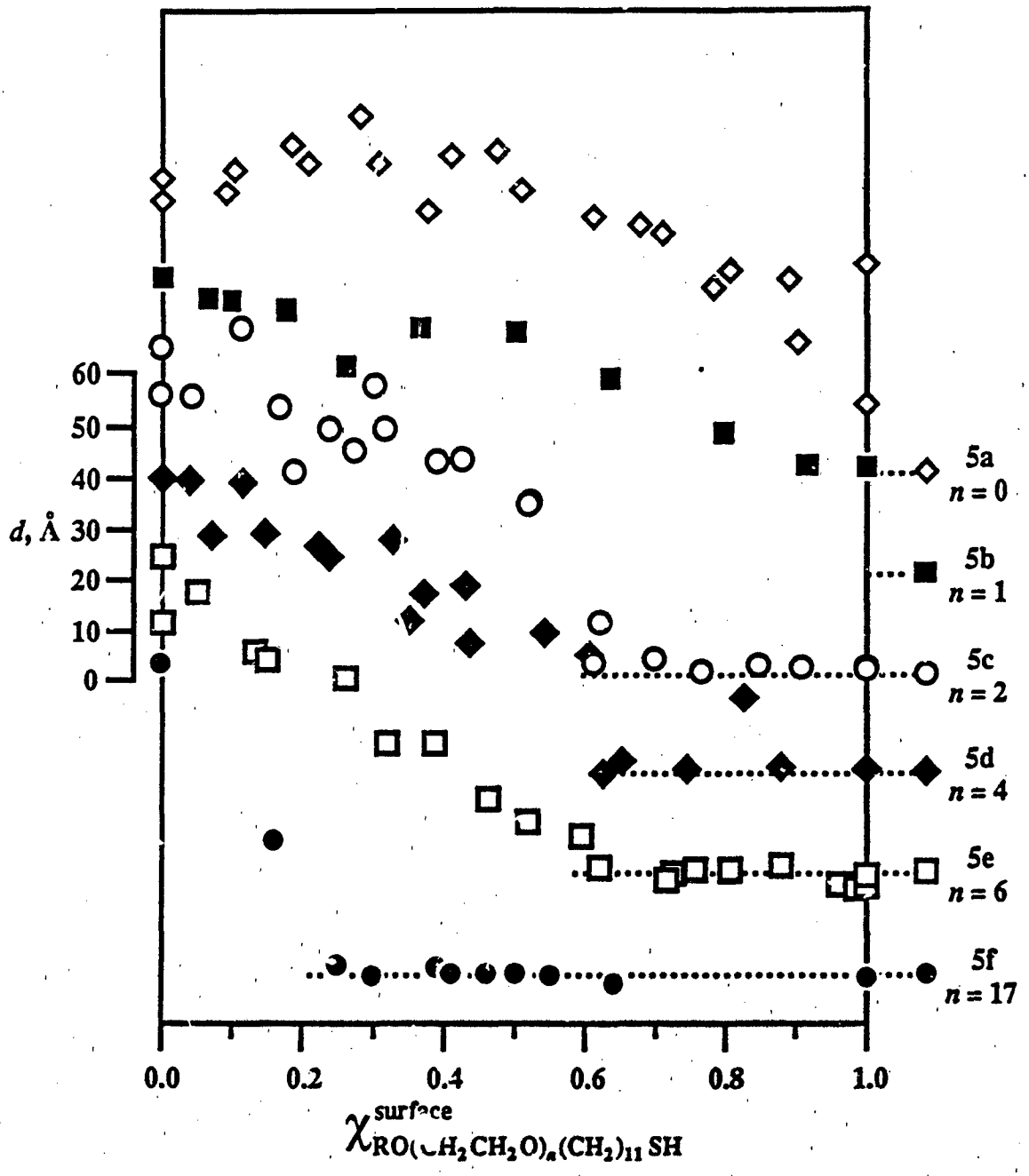


Figure 5. Adsorption of fibrinogen to SAMs formed from mixtures of 4 and 5.

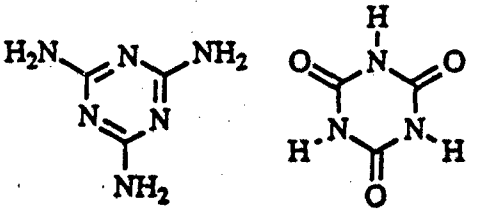
Second, adsorption of fibrinogen to mixed SAMs containing chains terminated by shorter oligomers of ethylene glycol (n = 2–6) reaches a minimum value within experimental error of the same concentration of oligo(ethylene glycol) chains in the SAM ($\chi \approx 0.65$). This observation suggests the possibility that a uniform, nonadsorbent interfacial structure is reached by all these SAMs around

$\chi = 0.65$, and that the thickness of this structure is not an important parameter in determining the adsorption of protein to the SAM. These data suggest that the elimination of protein adsorption on polymer surfaces by end-grafted poly(ethylene glycol) chains depends more upon the grafting density than upon the length of the grafted chain (*37, 38*).

These SAMs are models for polymer surfaces. They should make it possible to analyze the relative importance of the effects at the solid–water interface influencing protein adsorption, and perhaps in time, to develop materials with selective adsorptivities for use *in vivo*.

Self-Assembly in Three Dimensions: New Classes of Non-Covalent Macromolecules. Hydrogen bonding is a principal source of the enthalpic driving force used both for molecular recognition and for self-assembly in biological systems. Perhaps the best-known example is the pairing of the bases in polynucleotides.

In an analogous non-biological system, a remarkably stable, solid, 1:1 complex of melamine (M, **6**) and cyanuric acid (CA, **7**) forms spontaneously when aqueous solutions of the two compo-



6 **7**

nents are mixed together (*39–41*). Figure 6 shows an idealized section of the polymeric network that is produced; the structure shown is not proven unequivocally. For comparison, the inset shows the adenine–uracil base pair.

We have been developing this three-dimensional self-assembling system into two new classes of compounds. The first is a series of ribbon-like polymers prepared from derivatives of **6** and **7** where one or more of the N–H bonds is replaced with an N–alkyl bond (*4*). The alkyl substituents strongly affect the solid-state structure of these ribbons. Figure 7 shows the X-ray structure of diphenylmelamine–diethyl barbituric acid (**8–9**) complex. We are currently attempting to obtain

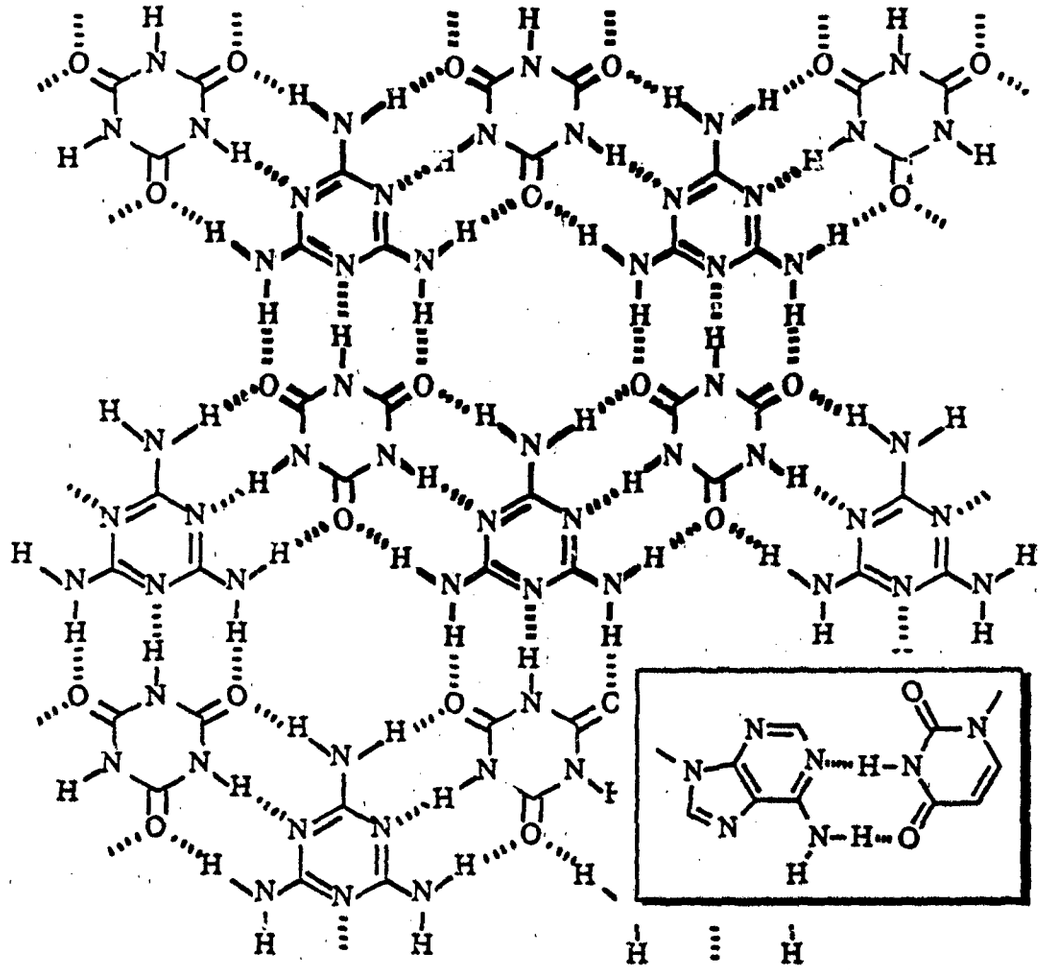


Figure 6. An idealized structure of the 1:1 complex between melamine and cyanuric acid (6–7). The inset shows the adenine–uracil base pair.

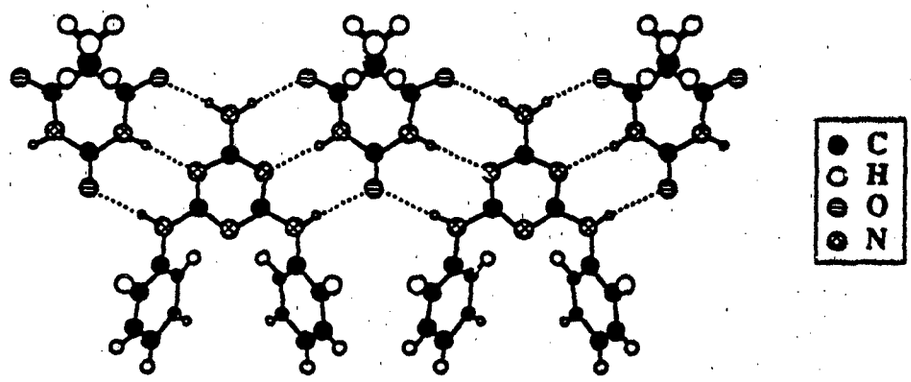
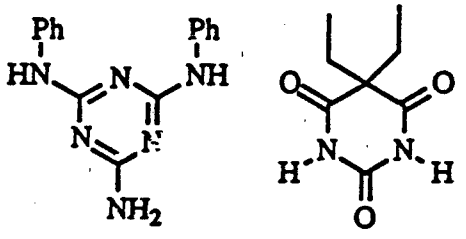


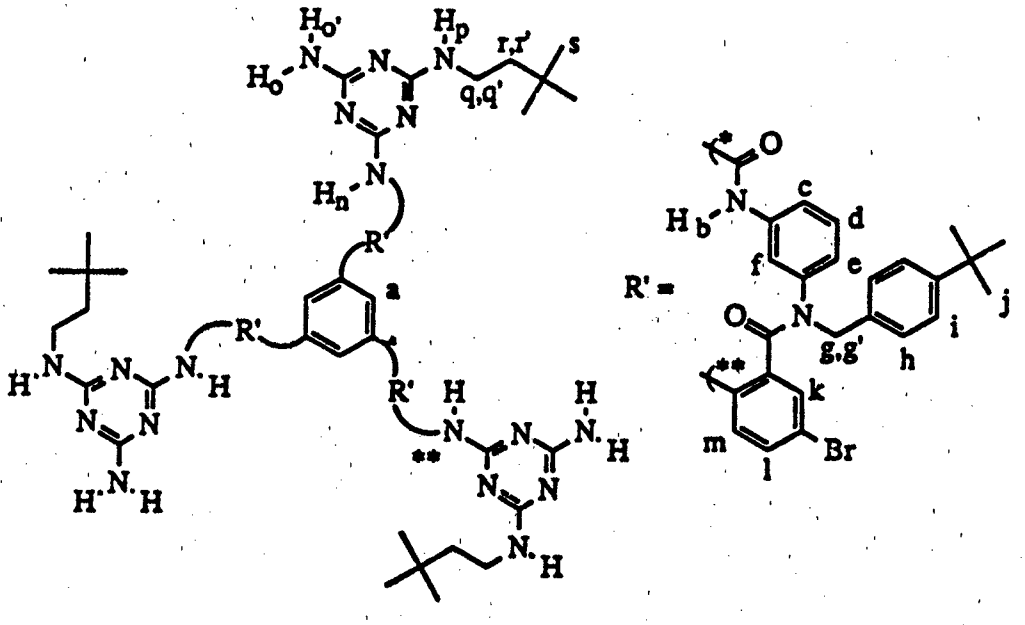
Figure 7. An X-ray crystal structure of the polymeric diphenyl melamine–diethyl barbituric acid (8–9) complex.

a predictive correlation between the structure of the alkyl substituent and the solid-state structure of the hydrogen-bonded polymer. For other approaches to the design of the solid state, see references *42–44*.

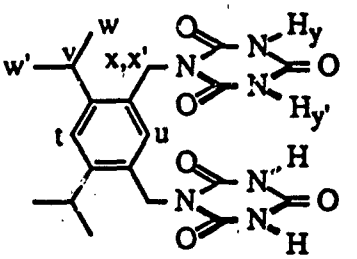


8 **9**

The second system involves the formation of smaller hydrogen-bonded complexes of a single structure (*5*, *6*). (For other supramolecular complexes, see references *45–47*.) Using molecular models, we predicted that the compounds we call hubM$_3$ (**10**) and R(CA)$_2$ (**11**) would self-assemble into a discrete 2:3 complex (Figure 8). We synthesized hubM$_3$ and R(CA)$_2$ and found that the product that resulted from their mixture was, indeed, the 2:3 complex. Figure 9 shows an NMR titration of hubM$_3$ with R(CA)$_2$: as R(CA)$_2$ is added, the three broad peaks arising from hubM$_3$ disappear and are replaced by sharp peaks arising from the complex. Exchange between the complex



hubM$_3$, 10



$R(CA)_2$, 11

and $hubM_3$ in solution is slow on the NMR time scale. We believe that the small peaks in the baseline of the upper spectrum correspond to conformational isomers of the 2:3 complex. These minor peaks are not impurities in either of the individual components. $R(CA)_2$ alone is too insoluble to give a detectable spectrum at saturation (< 0.1 mM) in $CDCl_3$ at the instrument gain used here. The NOE spectra, ultraviolet titrations, and molecular weights obtained from vapor-pressure osmometry are all consistent with the formation of a 2:3 complex.

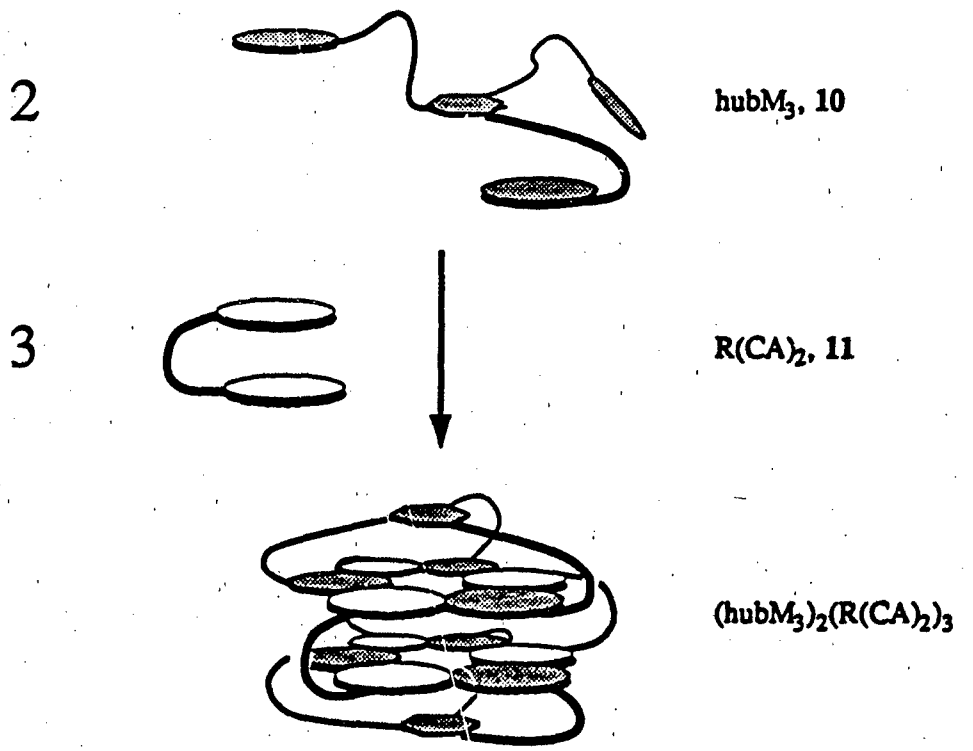


Figure 8. A schematic representation of the self-assembly of $hubM_3$ (10) with $R(CA)_2$ (11) to give a supramolecular complex. (Adapted from ref. 6. Copyright 1991 American Chemical Society.)

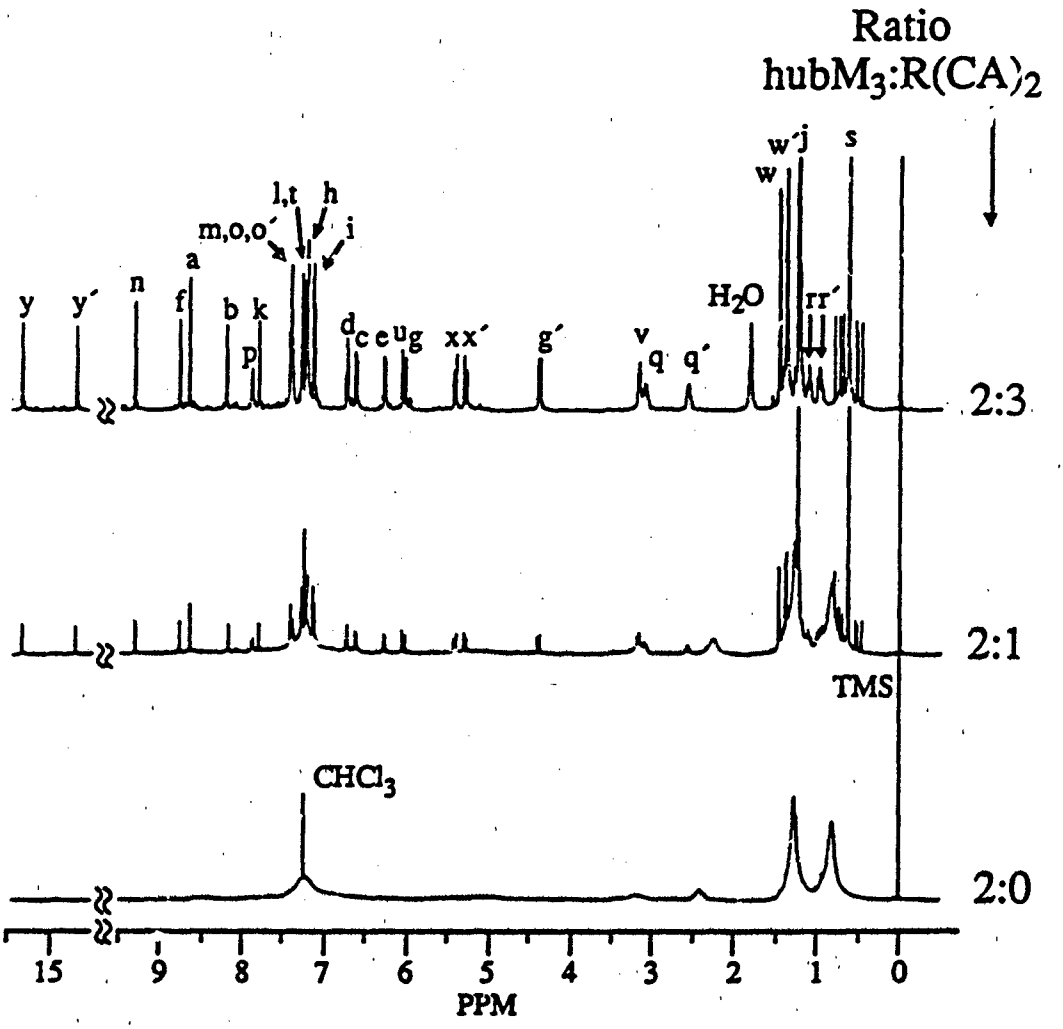


Figure 9. ^{1}H NMR titration of hubM$_3$ (10, 500 MHz, 10 mM in $CDCl_3$) with R(CA)$_2$ (11). The peak assignments are shown at the top of the figure and correspond to the labels on the structures of 10 and 11 shown in the text. (Reproduced with permission from ref. 6. Copyright 1991 American Chemical Society.)

Conclusion

Studies of molecular recognition and of synthetic polymers combine in a number of useful approaches to new analytical tools, to drug design, and to new materials. Polymers permit the interactions provided by the structural elements used for molecular recognition to be localized, addressed, and amplified through cooperativity or polyvalency. The synthetic methodologies available from polymer science provide convenient routes to complex, multifunctional substances;

molecular recognition and self-assembly provide new strategies for the synthesis of high molecular weight assemblies.

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